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Immunity against extracellular pathogens

John Gatfield, Giorgio Ferrari, and **Jean Pieters***

Basel Institute for Immunology, Basel

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Summary. Eukaryotic cells live in a relatively comfortable equilibrium with a wide variety of microbes. However, while many of the cohabiting microorganisms are harmless or even beneficial to the eukaryotic host, a number of prokaryotes have evolved the capacity to invade and replicate within host cells, thereby becoming potentially pathogenic. To be able to cope with potential pathogens, most organisms have developed several host defense mechanisms. First, microbes can be internalized and destroyed by a number of cell types of an innate immune system in a rather aspecific manner. Second, more complex organisms possess additionally an adaptive immune system that is capable of eliminating hazardous microbes in a highly specific manner. This review describes recent progress in our understanding of how pathogens interact with cells of the immune system, resulting in activation of the immune system or, for certain microorganisms, in the evasion of host defense reactions.

Keywords: Antigen processing; Antigen presentation; Phagocytosis; Host defense subversion.

Introduction

In the fight against microbes, various types of host responses occur, which fall into two classes. First, several nonspecific host defense mechanisms exist (innate immune response), and second, the host possesses various mechanisms that are directed at a particular invader (adaptive immunity). Under normal circumstances, the first line of defense is provided by the cells of the innate immune system, the macrophages, neutrophils, dendritic cells, and natural killer cells. These cells are attracted to the site of infection through multiple stimuli; macrophages and neutrophils accumulate at these sites in response to bacterial products, while cytokines secreted by these cells attract more macrophages and neutrophils as well as dendritic cells and natural killer cells. The innate immune system ensures an effective response by efficiently degrading the microbes (antigens) either through intracellular proteolysis following uptake or via the release of degradative enzymes after release of their granule content (Brown et al. 1994, Janeway 1989).

A more specialized response to invading pathogens is provided by cells of the adaptive immune system, comprising the B cells (originating in the bone marrow, hence the name B lymphocytes) and the T cells (originating in the thymus, therefore termed T lymphocytes). Importantly, activation of T lymphocytes depends on the recognition by the T cell receptors of peptides derived from pathogenic organisms. These peptides are not recognized alone, but in a complex with specialized molecules, the class I and class II molecules encoded by the major histocompatibility complex (MHC) (Zinkernagel 1997).

Presentation of microbial antigens to the immune system

The two classes of peptide presenting molecules (MHC class I and class II molecules) have probably evolved as a result of the existence of two types of pathogens that can invade the eukaryotic cell. One type, such as viruses, occupies the cytoplasmic compartment, and viral peptides are presented by MHC class I molecules. The other type, most extracellularly residing bacteria, enters the endosomal-lysosomal organelles, and bacteria-derived antigens are being presented by MHC class II molecules. An overview of the MHC class I and class II pathways is provided in Fig. 1.

* Correspondence and reprints: Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel, Switzerland.

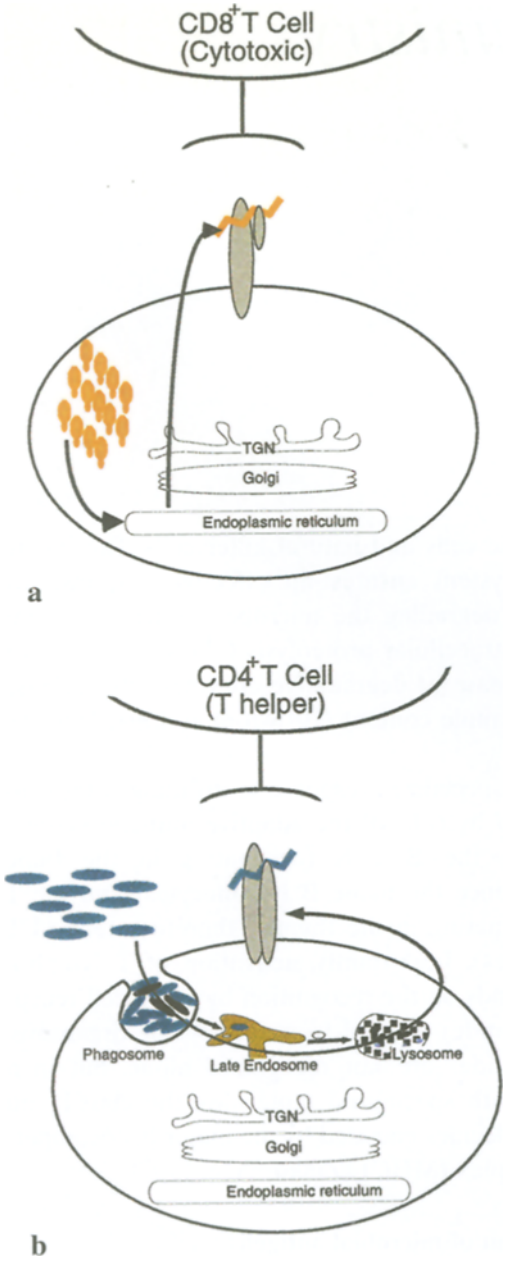

Fig. 1a, b. The MHC class I and class II pathways. **a** MHC class I molecules acquire antigenic peptides that are generated from infectious agents residing in the cytosol of infected cells (by, e.g., viruses). These peptides are formed by the proteasome and then translocated into the endoplasmic reticulum by the TAP complex. The MHC class I-peptide complex is transported through the biosynthetic pathway directly to the plasma membrane, for the activation of $CD8^+$ T lymphocytes. **b** Peptides to be presented by MHC class II molecules are usually derived from antigens that are present extracellularly (such as most bacteria). This extracellularly residing material enters the cell through endocytosis or phagocytosis and is degraded within endosomal-lysosomal organelles. The resulting peptides are loaded onto MHC class II molecules in specialized organelles, the so-called MHC class II compartments, prior to transport of the class II-peptide complex to the cell surface for triggering of $CD4^+$ T helper lymphocytes (see also Janeway and Travers 1996)

Both MHC class I and class II molecules, being transmembrane molecules, are synthesized on endoplasmic-reticulum-associated ribosomes and cotranslationally inserted in the endoplasmic-reticulum membrane (Dobberstein et al. 1979, Ploegh et al. 1979). The MHC-encoded molecules are generally highly polymorphic, ensuring that a wide variety of different peptides can be presented by these molecules (Benacerraf and McDevitt 1972, Kaufman et al. 1984, Mengle-Gaw and McDevitt 1985, Snell 1986, Trowsdale and Campbell 1992).

MHC class I pathway

MHC class I molecules are expressed by all cells and associate in the endoplasmic reticulum with another molecule, β-2-microglobulin (Heemels and Ploegh 1995). Proteins derived from viruses and bacteria that reside in the cytosol are degraded by the cytosolic proteolytic machinery, the proteasome, and the resulting peptides are transported into the endoplasmic reticulum by transporter molecules, the TAP molecules (transporter associated with antigen processing) (Deverson et al. 1990, Spies et al. 1990). MHC class I molecules become loaded with antigenic peptides in the endoplasmic reticulum and are then transported via the secretory pathway through the Golgi complex to the cell surface where they can be recognized by a subset of T lymphocytes, the cytotoxic T cells (Pamer and Cresswell 1998, Townsend et al. 1989). Triggering of T cell receptors on cytotoxic T cells usually results in the death of the antigen-presenting cell, thus effectively eliminating the source of the infection (see Fig. 1).

MHC class II pathway

Class II molecules, in contrast to MHC class I molecules, are expressed by only a subset of cells specialized in the presentation of extracellularly present antigens, including B cells, macrophages, and dendritic cells. Peptides destined to be presented by class II molecules are usually derived from exogenous antigens. Therefore, deposition of peptide-loaded MHC class II molecules on the cell surface of antigen-presenting cells involves not only organelles of the secretory pathway but also of the endocytic pathway (Pieters 1999a, Wolf and Ploegh 1995).

During the past decade, work from a variety of laboratories has delineated the pathways followed by MHC class II complexes. Directly after biosynthesis

and insertion into the membrane of endoplasmic reticulum, the class II molecules assemble noncovalently with a third molecule, the so-called invariant chain (Ii). In contrast to the class II molecules, the Ii is nonpolymorphic and in fact encoded outside the MHC (Koch et al. 1989, Machamer and Cresswell 1982). The Ii performs three important functions during the biogenesis of surface-deposited class II molecules. First, the Ii acts as a chaperone in the endoplasmic reticulum, to aid in the folding and the egress of the class II/Ii complex from the endoplasmic reticulum (Anderson and Miller 1992). Second, the Ii contains a sequence (termed CLIP, for class II-associated invariant-chain peptide) that binds directly to the class II peptide-binding groove, in a manner identical to antigenic peptides (Ghosh et al. 1995). In the endoplasmic reticulum, the Ii therefore shields the class II peptide-binding groove from binding peptides that should, as mentioned above, bind to the MHC class I molecules (Eynon et al. 1999, Roche and Cresswell 1990, Teyton et al. 1990).

Targeting of MHC class II molecules to the endocytic pathway

The third important function of Ii is to provide a targeting signal for the class II molecules to be transported to organelles of the endocytic pathway. Indeed, peptides destined to be presented by class II molecules are usually derived from exogenous antigens that have entered the endocytic pathway of the antigen-presenting cell (Cresswell 1994a, Pieters 1997b), and the molecule responsible for getting the class II complex into the endosomes is the associated invariant chain. The signals that are responsible for this targeting event reside within the cytoplasmic tail and consist of two di-leucine-based motifs (Bakke and Dobberstein 1990, Lotteau et al. 1990, Pieters et al. 1993). These signals mediate targeting of the class II complex from the trans-Golgi network to the endosomal pathway and, in addition, mediate the internalization of complexes that have arrived at the plasma membrane (Pieters et al. 1993).

The MHC class II compartments

Whereas initially the site where MHC class II molecules combine with antigenic peptides remained elusive, over the past few years the precise subcellular organelles to which class II/Ii complexes are targeted have been characterized relatively well. By immunocytochemistry on cryosections, it was shown that class II molecules, Ii as well as internalized material could be found within the same vesicles (Pieters et al. 1991). These organelles had a typical multivesicular morphology and were therefore referred to as multivesicular bodies (Pieters et al. 1991), or, because of their high class II content, MHC class II compartments (Peters et al. 1991, Pieters 1997a).

After the initial morphological identification of the MHC class II compartments, their subsequent biochemical isolation from various cell types allowed their in-depth characterization (Amigorena et al. 1994, Tulp et al. 1994, West et al. 1994). This work established the class II compartments as distinct organelles, separate from the endosomal-lysosomal pathway (Schmid and Jackson 1994).

More recently, it was established that the MHC class II compartments actually consisted of two physically and functionally distinct vesicle populations (Ferrari et al. 1997). Newly synthesized class II/Ii complexes travel through these organelles in a sequential manner: due to the endosomal targeting signals within the Ii cytoplasmic tail, the complex is first targeted to an organelle where the Ii is degraded from its C-terminal, lumenal side, resulting in a class II complex still associated with the CLIP peptide. The class II-CLIP complex, most likely due to a sorting signal within the class II molecules themselves, are then targeted to a second organelle, where CLIP is released from class II molecules. Interestingly, this latter compartment contains the HLA-DM molecules, which function as facilitators of peptide loading onto class II molecules (Cresswell 1994b; Mellins et al. 1990; Roche 1995, 1996). Indeed, peptides are readily loaded in this latter organelle, which therefore has been termed compartment of peptide loading (CPL) (Ferrari et al. 1997, Pieters 1999a) (see also Fig. 1). From the compartment of peptide loading, peptide-loaded class II molecules travel to the cell surface by as yet undefined mechanisms, where they can trigger T cell receptors on T lymphocytes leading to their activation. Activated T cells can then aid in the elimination of the invaded microorganism, by either promoting production of specific antibodies by B cells or by activating phagocytic cells to more efficiently ingest and destroy microbes.

Entry of microorganisms into eukaryotic cells

Thus far we have discussed the mechanisms involved in the transfer of antigens to the MHC molecules that

present these to the T lymphocytes of the immune system. But an important question is how these antigens end up in the appropriate cells. Extracellular pathogens, whose peptides are being presented by MHC class II molecules, usually enter the antigen-processing and -presenting cell via endocytosis. Several forms of endocytosis exist. First, soluble material (e.g., that released by the microbes) can enter the cell through pinocytosis ("cell drinking"; more recently renamed endocytosis), which is the vesicular uptake of fluid and macromolecules. Pinocytosis can be receptor mediated or occur via fluid-phase uptake, either in large ("macropinocytosis", i.e., the uptake of fluid into large, 0.15–0.5 μm diameter vesicles) as well as in small (ca. 100 nm diameter) vesicles. In addition, professional antigen-presenting cells such as the dendritic cells and the macrophages have the capacity to internalize entire microorganisms through the process of phagocytosis. Phagocytosis ("cell eating") refers to the internalization of large particles and microorganisms into large (≥250 nm diameter) vesicles.

The endocytic pathway

Regardless of the mode of uptake, material that is internalized enters the endocytic pathway, is transported from early to late endosomes, and may eventually reach lysosomes (Kornfeld and Mellman 1989). The molecular mechanisms involved in transfer of material through the endosomal-lysosomal pathway is only beginning to be unraveled. Whereas the processes governing receptor-mediated uptake are now relatively well understood, those involved in phagocytosis and fluid-phase endocytosis remain less well characterized and will be subject to review elsewhere in this series. Here, we will focus on the processes involved in entry and degradation of internalized microorganisms as well as microbial products.

During receptor-mediated endocytosis, receptors that are engaged by ligands can become clustered at the plasma membrane due to receptor cross-linking. During this process a scaffold protein, clathrin, is recruited to the cytoplasmic domain of the receptor and binds via a series of adaptor proteins that are specific for certain signals residing within receptor cytoplasmic portions (Kirchhausen et al. 1997, Pearse and Robinson 1990, Robinson 1994, Schmid 1997). The current model proposes that clathrin-containing coats initiate the plasma membrane invaginations containing receptor-ligand complexes at the cytosolic side of the plasma membrane and aid in the formation of clathrin-coated vesicles from the plasma membrane (Kirchhausen 1999, Sever et al. 1999). These clathrin-coated vesicles are then uncoated, and their content is transferred to consecutive stages of the endocytic pathway (Schmid 1997). Recent work suggests that at several levels of the endocytic pathway, sorting events may occur that are dictated by the presence of specific sorting determinants, processes that may be regulated through various coat proteins (Stoorvogel et al. 1996, Whitney et al. 1995). Sorting of proteins within the endocytic pathway may lead to transport of proteins either back to the plasma membrane, to the trans-Golgi network, to late endosomes, or to lysosomes (Gu and Gruenberg 1999, Keller and Simons 1997, Kirchhausen et al. 1997).

Phagocytosis

Phagocytosis is defined as the internalization of entire microorganisms or relatively large particles rather than small molecular compounds. As a consequence, the mechanisms and machinery involved in these processes are likely to be distinct from those involved in endocytosis. During phagocytosis, relatively large pieces of the plasma membrane that surround the microorganism are internalized. Subsequently, this phagocytosed material has to be transferred to later stages of the endocytic pathway, followed by delivery to lysosomes (or formation of a so-called phagolysosome through fusion of nascent phagosomes with lysosomes) where this material can be degraded.

A number of receptor molecules have been implicated in the internalization of microbes by phagocytes and other cells. These include Fc receptors, mannose receptors, scavenging receptors, and various integrins (Brown 1991, Daeron 1997, Ezekowitz et al. 1991, Fanger et al. 1996). Furthermore, several microorganisms have developed sophisticated mechanisms to gain entry into eukaryotic cells (Falkow 1991, Finlay and Cossart 1997). Some of these make use of host cell components, for example in the case of lysosome recruitment by trypanosomes (Tardieux et al. 1992), or express proteins that function as a bona fide receptor for a eukaryotic-cell surface component. Examples of these are the invasins expressed by *Escherichia coli* or *Yersinia pseudotuberculosis* and binding to integrins (Isberg and Leong 1990, Leong et al. 1995); internalin expressed by *Listeria* spp. and binding to E-cadherin (Mengaud et al. 1996); Tir expressed by enteropatho-

genic *E. coli* (EPEC), which is directly inserted into the host plasma membrane (Kenny et al. 1997, Rosenshine et al. 1996), and the type III secretion mechanisms present in, e.g., *Salmonella* spp., *Shigella* spp., and *Yersinia* spp., that generate a translocation system that is inserted into the host plasma membrane and interacts with the host cytoskeleton (Bliska et al. 1993, Cornelis 1998, Galan and Bliska 1996). Thus, it seems that many different pathways exist to internalize a microorganism into phagocytes; the precise mode of uptake may have evolved as a result of the long-term coevolution of microbes with phagocytic cells (Falkow 1991).

Macrophage activation

One result of phagocytosis of particulate material as well as living microorganisms is activation of the macrophages. Whether or not this activation is similar to the above mentioned T-cell-induced macrophage activation is not clear. In either case, the overall effect of macrophage activation is an enhanced capacity of the cell to inactivate and degrade phagocytosed microorganisms as well as cellular material including tumor cells (Adams and Hamilton 1992, Crawford et al. 1994). In addition, a plethora of cytokines that are released at the site of infection upregulate expression of MHC class II molecules and can also activate the T and B lymphocytes of the acquired immune system, thereby contributing to elimination of the infection.

The cell biological processes responsible for and accompanying macrophage activation are still poorly understood. Most likely, multiple signals are involved in generating an "activated macrophage", and which signals dominate may depend on the location of the macrophage within the organism, the type of infection, and the presence of accessory cells that can secrete activating cytokines. Whereas virtually every known cytokine has been implicated in macrophage activation, the best studied is gamma interferon (IFN-γ). INF-γ, when added to macrophages, upregulates MHC molecules, enhances phagocytosis and for some microorganisms results in an increased killing (Fowles et al. 1973). IFN-γ does not fully activate macrophages, but rather induces a "primed" state (Adams and Hamilton 1992). Primed macrophages can then be fully activated by other compounds such as low amounts of lipopolysaccharide (LPS, also called endotoxin), a glycolipid that is a component of the gram-negative bacterial cell wall (Adams and Hamilton 1984). LPS binds to a macrophage cell surface receptor and thereby activates a signal transduction cascade resulting in the secretion of various cytokines that together contribute to the activation of the macrophage. Very recently, the receptor that is activated upon LPS addition to macrophages has been determined to be a member of the Toll-like receptors (TLR) (Kirschning et al. 1998, Poltorak et al. 1998, Yang et al. 1998). These receptors bear homology to Toll, a molecule conserved from plants to mammals, and are thought to play a role in host defense (Fearon 1997, Lemaitre et al. 1996, Medzhitov et al. 1997).

LPS does not bind directly to Toll-like receptors but does so via the GPI(glycosyl phosphatidylinositol)-linked cell surface molecule CD14 (Gerard 1998). LPS that is shed from the bacteria within a host usually aggregates, and these aggregates bind to a serum protein called LPS-binding protein (LBP). LBP breaks down these aggregates allowing the binding of LPS to the cell surface molecule CD14. But because CD14 lacks a transmembrane and cytosolic domain (being a GPI-linked molecule), it cannot directly transmit activation signals to the cytosol. The molecules that seem to be responsible for transmitting signals are the aforementioned TLRs, which are activated upon engagement by LPS in the presence of LBP and CD14 (Gerard 1998; Medzhitov and Janeway 1997, 1998; Medzhitov et al. 1997; Yang et al. 1998).

Apart from LPS, virtually every known cytokine has been shown to act in macrophage activation, alone or in concert with other reagents, and to varying efficiency with regard to microbicidal activity against different microorganisms (Crawford et al. 1994). However, it is currently unknown which components induce activation in vivo. The availability of mice lacking one or several cytokines and/or cell surface receptor molecules may help to elucidate the precise molecules involved.

Macrophage activation leads to several cellular processes that aid in the elimination of the microorganisms. First, activated macrophages upregulate their phagocytic capacity. Second, the capacity of macrophages to kill the phagocytosed microbes is drastically enhanced, through the upregulation of radical formation in the phagosome on the one hand, and through the stimulation of phagosome-lysosome fusion on the other. Third, activated macrophages upregulate the expression of MHC class II molecules, allowing the adaptive immune system to contribute to the elimination of the invading microorganisms.

Evasion of host defense mechanism

The microbicidal activities of macrophages, together with the capacity of the adaptive immune system to generate a strong antibody-mediated response, is usually sufficient to eradicate any microbe that has invaded the host organism. However, many microorganisms have coevolved with their eukaryotic host for a considerable time, and it is therefore not surprising that various microbes have developed mechanisms allowing them to circumvent host defense responses. In fact, one reason why some microbes have become pathogenic is precisely because they have evolved ways to survive and replicate within their mammalian host. Many pathogens do this by finding a suitable site within the host cell where they can thrive and at the same time circumvent host defense mechanisms (Falkow et al. 1992, Galan and Bliska 1996, Pieters 1999b, Small et al. 1994).

Subversion of phagocytosis by mycobacteria

One particularly successful example of pathogen–host interaction is provided by the interaction of mycobacteria with macrophages. Macrophages, as mentioned, are among the most professional phagocytes, which, in addition, can present antigenic peptides bound to MHC class II molecules to T lymphocytes (Silverstein 1995, Unanue 1984). Indeed, macrophages do ingest mycobacteria and are also able to stimulate T lymphocytes to a certain degree (de Chastellier et al. 1995, Inaba et al. 1993, Kaufmann 1993). But at the same time, mycobacteria have the capacity to resist destruction by the macrophage, and as a result, these bacilli can survive and even replicate within the macrophage (Russell 1995). In doing so, mycobacteria can also become sequestered from the activity of T lymphocytes, thus allowing these bacteria to escape immune defense mechanisms (Pancholi et al. 1993). These highly efficient escape mechanisms are believed to be involved in the pathogenicity of mycobacterial infections, which include tuberculosis, leprosy, as well as various opportunistic infections (Bloom 1992, Murray and Salomon 1998).

The mechanism by which mycobacteria resist destruction in the macrophage is linked to the capacity of these bacteria to inhibit phagosome-lysosome fusion (Armstrong and D'Arcy Hart 1971, Barker et al. 1997, Hasan et al. 1997). The molecular basis of this capacity has however remained unclear until recently, when it was demonstrated that within macrophages, living mycobacteria recruit and retain a host protein, TACO (tryptophane aspartate-containing coat protein). As a result of this retention, the mycobacterial phagosome is unable to fuse with or mature into lysosomes (Ferrari et al. 1999). In contrast, killed mycobacteria cannot retain TACO and are therefore rapidly delivered to lysosomes followed by their degradation (see Fig. 2). Although the normal cellular function of TACO is still unknown, it might be involved in generating the forces that are needed to internalize large particles, including (living) bacteria. Upon phagocytosis, TACO forms a coat around the phagosome that has to be released to allow the fusion machinery to be recruited at the phagosomal membrane. Apparently, during the long-standing coevolu-

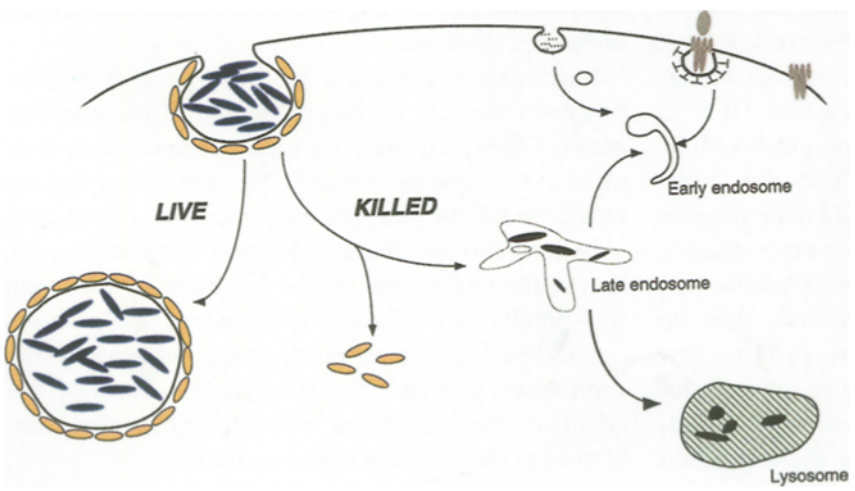

Fig. 2. Evasion of macrophage host defense mechanisms by mycobacteria. Phagocytosis of mycobacteria (blue) into macrophages triggers the recruitment of TACO (yellow) around the nascent phagosome. In case of killed mycobacteria, the TACO coat has to be removed in order to allow delivery of the phagosomal content to lysosomes, where this material is degraded. In contrast, living mycobacteria that have entered the macrophage have somehow gained the capacity to retain TACO at the phagosomal membrane, thereby preventing their delivery to lysosomes and allowing them to survive within the phagosome

tion of mycobacteria with their host organisms, they have managed to actively recruit and retain TACO for their own benefit.

By hiding inside macrophage phagosomes, mycobacteria not only manage to circumvent the normal bactericidal mechanisms of these cells but, in addition, become invisible for other host defense mechanisms, such as the generation of antimycobacterial antibodies that of course can only deal with extracellularly residing antigens. In addition, because of the nondegradative environment of the mycobacterial phagosome, antigenic fragments cannot be generated, preventing the generation of a proper MHC-dependent T cell response (Pancholi et al. 1993).

The strategy employed by mycobacteria to circumvent destruction within macrophages is once more an intriguing example of how microbes have evolved in concert with their host organisms. In addition, knowledge of these strategies might allow the design of rational agents to combat pathogens that escape the normal immune defense reactions.

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